Disulfide-Based Diblock Copolymer Worm Gels: A Wholly-Synthetic Thermoreversible 3D Matrix for Sheet-Based Cultures

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Disulfide-Based Diblock Copolymer Worm Gels: A New Wholly-Synthetic Thermo-reversible 3D Matrix for Sheet-Based Cultures

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Three-dimensional (3D) culture systems are attracting increasing attention, since, in principle, they can provide a microenvironment that resembles more closely that of tissue *in vivo*, than do conventional two-dimensional (2D) culture systems.[1, 2] The 3D microenvironment includes cell-cell and cell-extracellular matrix (ECM) interactions, which are both known to regulate signaling and differentiation of cells. The 3D structure also influences local gradients that govern mass transport of oxygen, glucose, metabolites, and signaling molecules.[2, 3] Measuring cellular viability and phenotype in hydrogel-based cell culture systems however, [4] often requires specialized histology[5] and/or optical techniques that restrict the widespread use of 3D cell culture in cell-based assays. Here, we describe the application of a new, disulfide-based, thermo-responsive diblock copolymer for embedding cells in mesh sheets for 3D cell culture; this gel persists for at least 12 days in culture. In aqueous solution, this diblock copolymer forms worm-like particles, which self-assemble

reversibly to form either entangled worm-like micelles that form a free-standing hydrogel at ambient temperature or spheres that form a free-flowing dispersion of de-gelled particles at sub-ambient temperature. Thus this diblock copolymer provides a convenient thermo-responsive matrix from which cells can be embedded and recovered without recourse to proteolytic enzymes such as trypsin. The efficiency of recovery of cells is comparable to that achieved via enzymatic degradation. A549-GFP cells released from such worm gels remain viable and can be further cultured or analyzed directly. Commercially available protein-based gels for cell culture (e.g. Matrigel, collagen, etc.) are expensive and must be stored at low temperature ($\leq$ 4 °C) to maintain stability and prevent degradation, whereas this wholly-synthetic hydrogel can be stored at room temperature, and still gel reversibly on demand.

Over the past two decades, hydrogels have emerged as a useful matrix to create 3D structures for either supporting or encapsulating cells *in vitro*.[6] For example, Bissell *et al.* demonstrated that malignant and non-malignant breast cancer cells can be distinguished on the basis of differences in morphology and gene expression when cultured in 3D using Matrigel[7] (a protein-based hydrogel extracted from Engelbreth-Holm-Swarm mouse sarcoma cells), but not when cultured in 2D.[8]

Hydrogels used for 3D cell culture can either be derived from natural biopolymers, [4, 9] or from synthetic polymers. [10] Protein-based hydrogels (e.g., Matrigel, collagen, silk), polysaccharides (e.g., hyaluronate, chitin) and polynucleotides (e.g., DNA, RNA), contain various bio-active species such as laminin, collagen, and entactin that promote cellular growth and signaling.[6, 11] Moreover, these components often vary in composition and concentration between batches, which can introduce artifacts in cell biology studies. [4, 12] Furthermore, such biopolymers have a limited shelf-life and are relatively expensive.[4] In contrast, synthetic hydrogels based on poly(ethylene glycol), polyacrylamide, poly(*N*-isopropyl acrylamide), poly(vinyl alcohol) or poly(acrylic acid)[10] have a user-defined composition and provide a cost-effective, reproducible and tunable environment for cell studies. These synthetic

hydrogels, however, lack the functionality required to promote biologically-relevant cell-matrix contacts. Furthermore, efficient harvesting of the embedded cells requires enzymatic, thermal, chemical, or optical disruption of the hydrogel cross-links. These degradation strategies can compromise cellular viability[9] and, in addition to other challenges associated with 3D cultures, have hitherto limited the applicability of such hydrogels in cell culture.[4]

Recently, RAFT aqueous dispersion polymerization has provided a facile route to a wide range of diblock copolymer nano-objects.[13] In particular, polymerization-induced self-assembly (PISA) of poly(glycerol monomethacrylate)-block-poly(2-hydroxypropyl methacrylate) (PGMA-PHPMA) diblock copolymers enables well-defined spheres, worms or vesicles to be produced at high solids, simply by targeting the appropriate diblock composition.[14, 15] The worm morphology is of particular interest, because these highly anisotropic particles form soft, free-standing aqueous gels.[15, 16] These worm gels are thermo-responsive: upon cooling to 5 °C, the hydrophobic core-forming PHPMA block becomes more plasticized by water, which induces a worm-to-sphere transition and causes de-gelling (i.e., separation of fused spheres). The resulting low-viscosity fluid is amenable to cold ultrafiltration, which provides a facile route to sterilization.[15] This thermal transition is fully reversible in semi-concentrated solution: one-dimensional fusion of multiple spheres lead to reformation of worms at 20 °C, which leads to rapid re-gelling. Small molecule impurities can be removed by dialysis, and mammalian cells embedded in the dialyzed worm gels remain viable. [15] Worrm gels—for a substantial number of reasons—thus seem to be a promising matrix for 3D cell culture.

Previously, we and others have demonstrated that sheets of paper or polymer-based mesh can be used to support cell-embedded gels ("Cells-in-Gels-in-Paper", CiGiP and Cells-in-Gels-in-Mesh", CiGiM).[17, 18, 19] Upon spotting with a micropipette, a cell suspension in Matrigel at 4 °C wicks through the sheets of paper or mesh. Upon incubation in media at

37 °C, the suspension forms a gel in the voids of the sheets. These systems provide a powerful method for manipulating and analyzing 3D cell cultures.[17, 18, 19]

Here we report the use of disulfide-functionalized PGMA-PHPMA worm gels (**Figure 1**) as an alternative to Matrigel for embedding cells in sheets of polymer-based mesh. This synthetic hydrogel offers several advantages: (i) the thickness of the cell culture can be controlled by choosing mesh sheets of appropriate thickness, (ii) the progress of cell growth and proliferation can be monitored in situ, since the mesh sheets allow transmission of light with minimal scattering, (iii) the cells can be harvested by simply immersing the sheets in cold buffer or media (instead of enzymatic degradation), and (iv) the gel can be heated and cooled reversibly without changing its physical properties, and thus does not require stringent storage conditions.

In preliminary experiments, non-functionalized PGMA-PHPMA [15, 16] worm gels were supported on either paper or a composite sheet of polyester mesh and poly(vinyl chloride) (see Supporting Information for further details). However, these platforms proved to be insufficiently robust: spotted PGMA-PHPMA worm gels detached from the sheets over 7-10 days (**Figure S1 and S2**). Therefore, we designed a second-generation worm gel that adhered more strongly to the mesh sheet; this new gel contained disulfide bonds within some of the PGMA stabilizer chains (**Figure 1A**), which were introduced using a strategy previously reported for disulfide-functionalized nano-objects.[20, 21] Briefly, a disulfide dimethacrylate (DSDMA) was statistically copolymerized with GMA via RAFT solution polymerization to produce a low-polydispersity poly(glycerol monomethacrylate)-stat-disulfide dimethacrylate ($PGMA_{55}$-$DSDMA_{0.50}$) macromolecular chain transfer agent (macro-CTA) (M_w/M_n = 1.26). This copolymerization was conducted in relatively dilute solution (10 % w/w) in order to suppress intermolecular branching, and hence favor intramolecular cyclization.[22] A 7:3 binary mixture of $PGMA_{54}$ and $PGMA_{55}$-$DSDMA_{0.50}$ macro-CTAs was

used for the subsequent RAFT dispersion polymerization of HPMA (**Figure 1A**). Further synthetic details are porvided in the Supporting Information.

Polymerization of HPMA using the binary mixture of $PGMA_{54}$ and $P(GMA_{55}$-stat-$DSDMA_{0.50})$ macro-CTAs at 20 % w/w solids produced a free-standing worm gel at 20 °C. After dilution to 8 % w/w solids, the dispersion remained a free-standing copolymer gel at 37 °C and transformed into a free-flowing fluid at 2 °C (**Figure 2A**). TEM images confirmed the presence of worms at the former temperature, and short worms and spheres at the latter temperature (**Figure 2A**). Rheology measurements indicated that the 8.0 % w/w worm gel exhibited an initial G' of around 20 Pa in PBS at 37 °C (**Figure 2B**).

To evaluate the effect of long-term storage, we incubated five identical worm gels at 37 °C, and then performed rheological measurements after 1, 2, 3, 4 or 5 days. The G' increased systematically from 20 Pa to 50 Pa (**Figure 2C**), which suggests the formation of inter-worm disulfide bonds (via thiol-disulfide interchange) as the gel aged at 37 °C.[21] We also monitored the changes in the properties of the de-gelled dispersions wer for gels stored at 37 °C for 5 days, then cooled to 2 °C. The graphs in **Figure 2B** (green data set) show (i) a slight shift in the critical gelation temperature (CGT) s to 13 °C, which indicates the weakening of the interaction of the resulting spheres, and (ii) an increase in G' from 0.03 Pa to 0.40 Pa, which suggests formation of additional inter-particle disulfide bonds. Nevertheless, the G' of the aged worm gel remained low and thus retained its thermo-reversible behavior.

Because thiol-disulfide exchange led to inter-worm cross-linking, we thus hypothesized that this mixed macro-CTA approach could produce worm gels that were strong enough to prevent worm gels from detaching prematurely from the supporting mesh sheet. To examine whether such disulfide-containing worms produced sufficiently strong gels within a sheet, [18] we spotted 1 μL suspensions of fluorescent 10 μm polystyrene microspheres into cold (2 °C) worm gels within zones of sheets of mesh or paper,[17, 23] and then immersed the

sheets in warm (37 °C) PBS for nine days. Fluorescence images revealed that the microspheres, and consequently the disulfide-functionalized worm gel, remained within the original zones (**Figures 2D, 2E and S1**). This result confirmed that functionalization with disulfide groups produces worm gels with increased robustness.

Unlike protein-based hydrogels such as Matrigel, synthetic worm gels lack the functionality (e.g., peptides such as RGD, growth factors, focal adhesion proteins, etc.) that promote cell growth.[4, 12] To determine whether cells embedded in these disulfide-based worm gels could still proliferate, we spotted 1 μL suspensions of A549-GFP cells (3×10^4 cells/zone) into worm gels within zones of the mesh sheets, and observed changes in cell density over the course of 12 days. Optical microscopy studies indicated that the density of A549-GFP cells increased progressively from the first to the fifth day of culture (**Figure 3A**). Cellular densities of A549-GFP cells became indistinguishable after 5 days of culture, so we imaged the cell-impregnated sheets with a fluorescence gel scanner to assess whether proliferation continued over longer time periods. Fluorescence intensities increased linearly over time (**Figure 3B**), which confirms that the cells remained viable and proliferative for at least 12 days while embedded within the worm gel.

Conventional cell culture methods require proteases (e.g., trypsin, Accumax, Accutase) to release cells from 2D surfaces or to recover cells from protein-based gels.[24] In contrast, cells cultured within thermo-responsive gels (e.g., poly(*N*-isopropyl acrylamide)-based gels)[25] can be isolated by simply cooling to liquefy the gel, thus releasing the cells from the gel matrix. To evaluate the latter strategy for worm gels, we incubated the mesh sheets containing A549-GFP cells embedded in worm gels in cold (4 °C) Dulbeccos's phosphate buffer saline (DPBS), and imaged the mesh sheets containing the cells immersed in worm gels using an optical microscope and a fluorescence gel scanner. Micrographs show that the cell-embedded worm gels detached progressively from the mesh after incubation in cold DPBS (**Figure 4A**). We estimated the percentage of cells retained in the mesh sheets by

measuring the fluorescence intensities from the zones as a function of time, and then calculating the reduction in fluorescence at each time point relative to the fluorescence of the zones before cooling. **Figure 4B** shows the reduction in GFP intensity over time: approximately 91 ± 6 % of the cells detached from the mesh sheets after 60 min in cold DPBS. GFP intensities determined for each time point indicates that the efficiency in the recovery of A549 cells in worm gels on cooling is comparable to that achieved when cells were recovered enzymatically from Matrigel (**Figure 4B**).

The recovery of the cells from the gel required using non-physiological conditions (i.e. incubation in cold media), which could potentially compromise cellular viability. To determine whether the recovered cells remained viable, we suspended A549-GFP cells (isolated either from worm gels or Matrigel) in culture media, dispensed the suspensions (~2,000 cells/zone) in 96-well plates, cultured for seven days, and measured the luminescence from the reaction of ATP (indicating metabolically-active cells) at various time points using CellTiter-Glo® (CTG) assay. Assay. We estimated the viability of the recovered cells over time by calculating the ratio of the luminescence intensity at each time point relative to the luminescence intensity measured at the start of the culture (24 h after dispensing the recovered cells in the well plates). The cellular density and normalized ATP levels of A549-GFP cells recovered from the worm gels increased monotonically up to a week after recovery (**Figures 4C and 4D**), which indicates good viability. Cells recovered from worm gels, however, proliferate less than those recovered from Matrigel (**Figure 4D**). This difference most likely reflects the fact that unlike Matrigel, the worm gels lack the various proteins and growth factors that are known to promote cellular growth and proliferation.[4] [12, 26]

Sheet-supported 3D cell culture provides a convenient means of handling and analyzing 3D cell cultures, and thermo-responsive hydrogels provide a convenient vehicle to deliver and embed cells into the sheets. The disulfide-functionalized diblock copolymer hydrogel described herein is expected to be preferable to commercial protein-based gels,

particularly for applications where the biological effects of such animal-derived gels are not acceptable, or are too variable.[27] This new synthetic hydrogel permits 3D culture of cells supported in mesh sheets and can be used to evaluate the effects of cell-ECM proteins for at least 12 days. In principle, thiol-disulfide chemistry can be used for convenient hydrogel functionalization with RGD, DNA or adhesion proteins to evaluate how these biomolecules influence cellular growth and proliferation. Such second-generation hydrogels should enable the effects of the bio-active species to be decoupled from the effect of growth factors that are typically present in protein-based gels.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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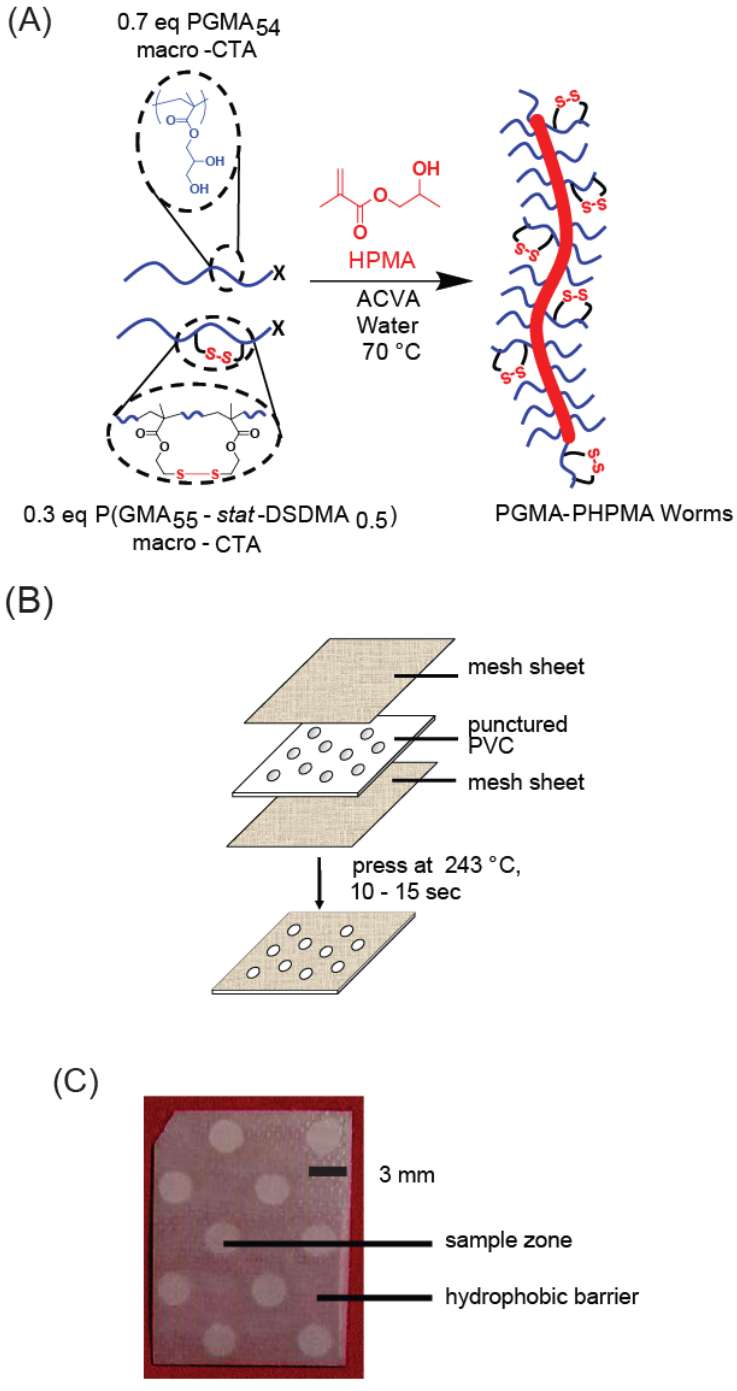

Figure 1. Schematic illustrations of (A) the synthesis of disulfide-functionalized PGMA-PHPMA worms via RAFT aqueous dispersion polymerization of HPMA at 70°C, and (B) fabrication of PVC-mesh composite sheets. (C) Photograph of a multi-zone mesh sheet.

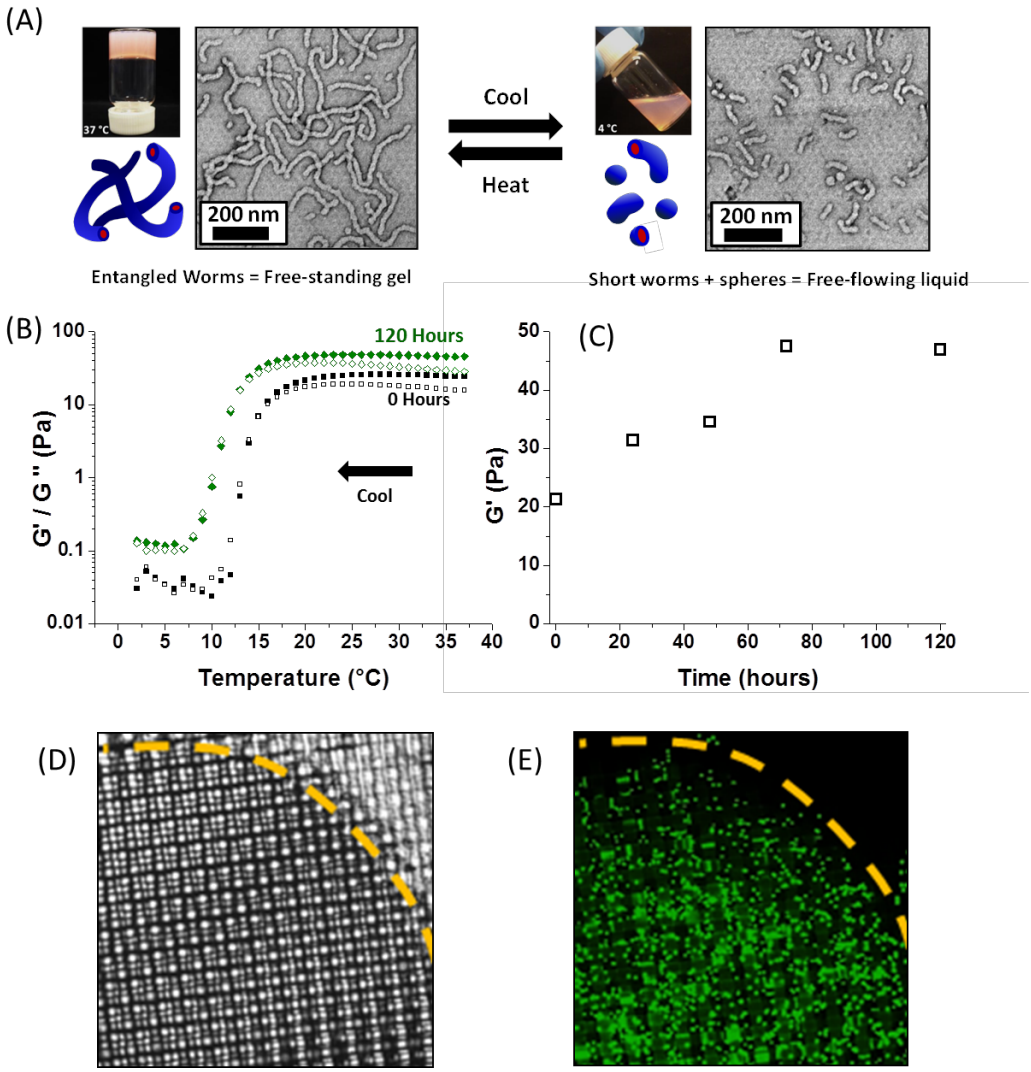

Figure 2. (A) Digital photographs and TEM images obtained at 37 °C and 2 °C showing the temperature-dependent (de)gelling behavior of the disulfide-functionalized copolymer worms. (B) Temperature-dependent oscillatory rheology measurements conducted on two identical 8.0 % w/w disulfide-functionalized copolymer worm gels before and after incubation in PBS at 37°C for 120 h. (C) Variation of the storage modulus (G') with ageing time for a series of five identical 8.0 % w/w disulfide-functionalized copolymer worm gels incubated for up to five days at 37°C. (D) Optical and (E) fluorescence microscopy images showing a portion of a zone of a sheet of PVC- mesh composite containing 10-µm fluorescently-labeled polystyrene particles embedded in a 10 % w/w disulfide-functionalized copolymer worm gel. Images were obtained nine days after immersion in 0.15 M PBS solution at 37°C. The retention of the fluorescein-labeled particles in the mesh indicates that the gel remains stable nine days after impregnating and gelling the suspension in the PVC-mesh composite sheets.

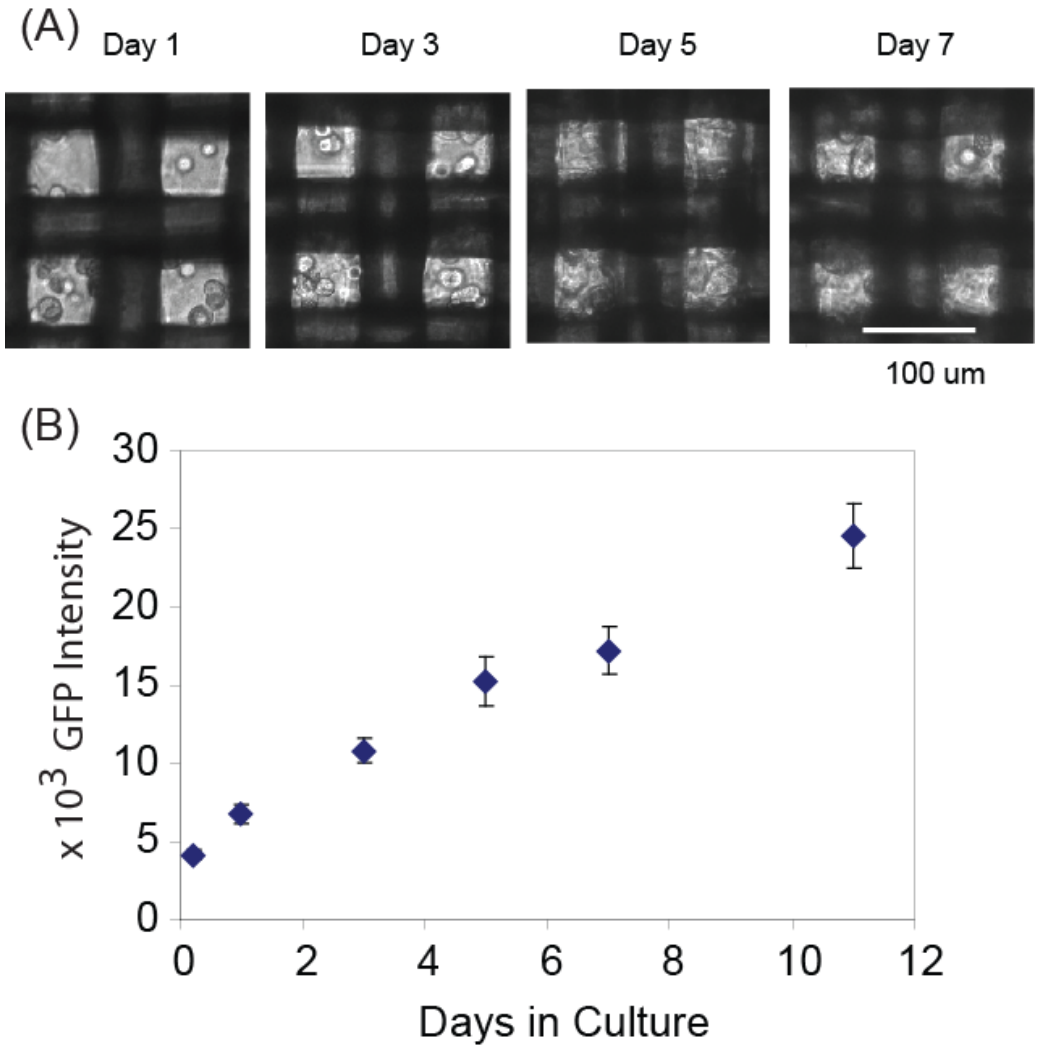

Figure 3. Worm gels allow A549 cells to remain viable within mesh sheets for up to 12 days. (A) Bright field images of A549 embedded worm gels supported on the PVC-mesh composite sheets. (B) Growth curve of A549-GFP-embedded worm gels supported on PVC-mesh composite sheets. Standard deviations were calculated based on seven replicates (N =7 zones).

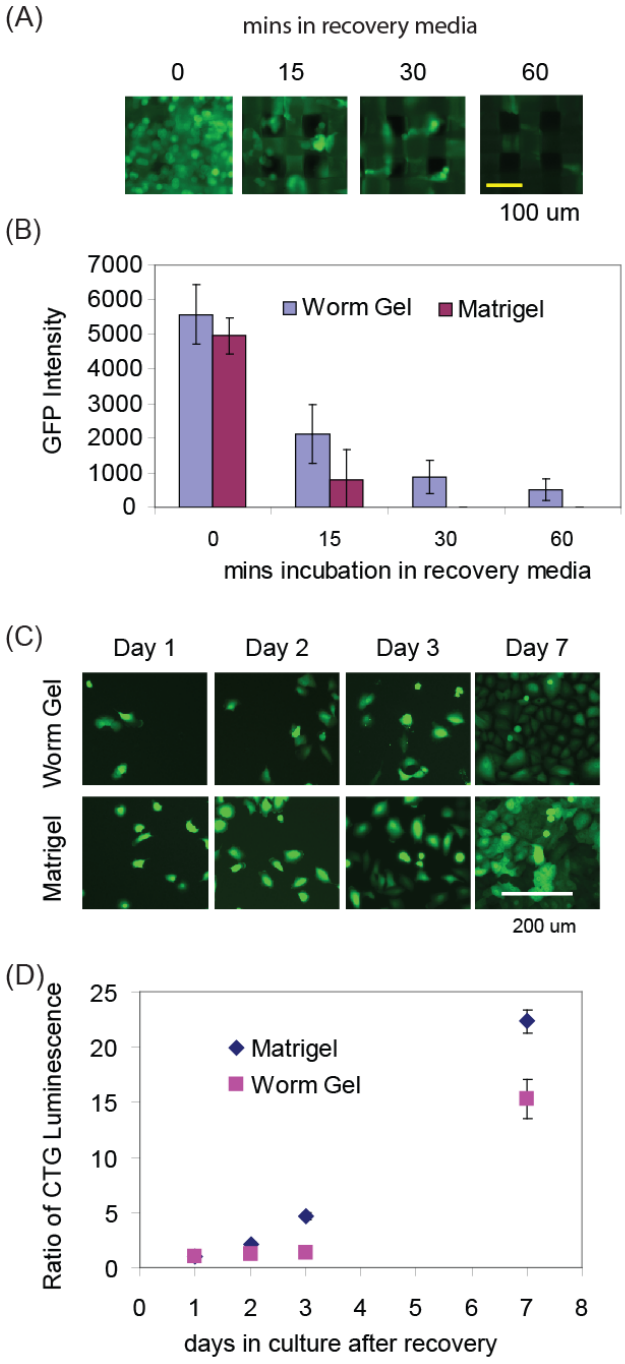

Figure 4. Recovery of A549-GFP cells from mesh sheets. Mesh sheets containing A549-GFP cells embedded in worm gel were incubated in cold PBS (4°C), while samples embedded in Matrigel were incubated in warm Accumax (37°C). (A) Fluorescence images of mesh sheets containing worm gel-embedded A549-GFP cells during recovery in cold PBS. (B) Extent of removal (as judged by normalized % GFP intensity) of A549-GFP cells embedded in either 10 % w/w disulfide-functionalized copolymer worm gel or Matrigel. Standard deviations were calculated based on thirty replicates (N = 30 zones). (C) Fluorescence images of 2D cultures of recovered A549-GFP cells. (D) Comparison of cellular viability of A549-GFP cells after recovery from worm gel or Matrigel, respectively. Recovered cells were suspended in media, seeded in a 96-well plate (2,000 cells/zone), and ATP levels were measured using CellTiter-Glo® (CTG) Assay. Luminescence was normalized to the first day of recovery. Standard deviations were calculated based on five replicates (N = 5 wells).

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Three-dimensional (3D) cell culture can provide *in vivo*-like microenvironment for cells *in vitro*, but the complexity in handling and analysis of cells in this system limit its widespread use. We demonstrate thermo-responsive disulfide-based copolymer as a matrix for sheet-based 3D cell culture. Unlike most gels for cell culture, this gel does not require refrigeration, nor enzymes to recover cells.

Keywords: 3D cell culture, sheet-based cell culture, hydrogels, thermos-responsive gels

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